Biological Report 17
July 1993

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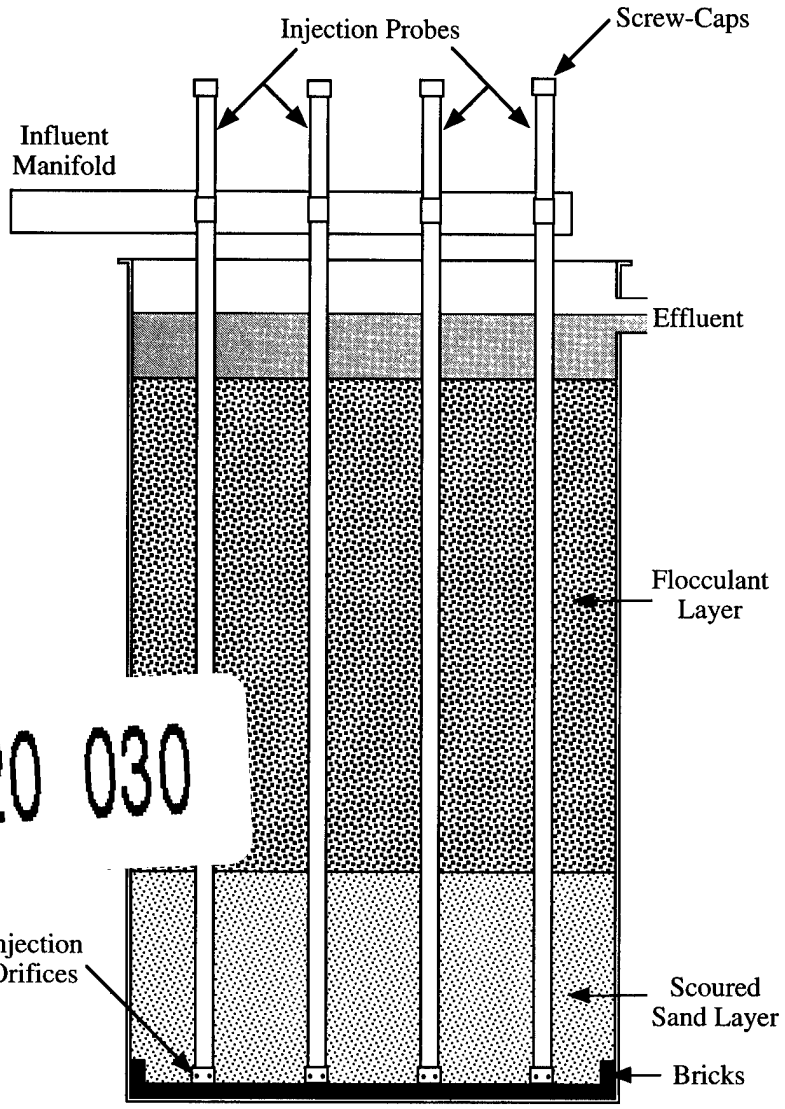

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By

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Qualitative and Quantitative Bacteriological Studies on a Fluidized Sand Biofilter Used in a Semiclosed Trout Culture System[1]

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Abstract. We conducted a study of the numbers and types of heterotrophic bacteria that occur in a fluidized sand biofilter and in rearing water in a semiclosed trout culture research system at the Freshwater Institute, Shepherdstown, West Virginia. Methods were developed for sampling the biofilter at various depths, removal of bacteria from the biofilm on sand particles, selection of appropriate media for enumeration of bacteria, and plate count procedures. Sonication of sand from biofilters and a spread plate technique provided the best results in determining total counts of heterotrophic bacteria. Total bacterial counts on sand at the top of the fluidized sand filter were $6.6 \times 10^7 - 7.0 \times 10^8$ compared with $2.4 \times 10^7 - 2.6 \times 10^8$ colony-forming units per gram of wet-packed sand at the bottom of the filter. Total heterotrophic counts in biofilter water and in trout-rearing water were similar, ranging from 10^4 to 10^5 colony-forming units per milliliter of water. Fluctuations of total counts on sand at the top and bottom of the biofilters were correlated with the concentration of ammonia nitrogen in water leaving the biofilter; as ammonia levels rose counts decreased, and as ammonia levels decreased

[1] This was a cooperative project among the Fish and Wildlife Service, Freshwater Institute, and Agricultural Research Service. It was funded through Agricultural Research Service Grant 59-1931-8-111.

counts increased. Identification of 274 bacterial isolates from biofilters and rearing-tank water showed that 77.1–83.8% were Gram-negative, nonfermenting, rod-shaped bacteria, and as much as 50% of those isolates were yellow-pigmented bacteria. Identification of isolates to the genus or species level was difficult even though three different identification schemes were used. Predominate isolates were identified as *Pseudomonas* or *Pseudomonas*-like, *Empedobacter* or *Empedobacter*-like, *Moraxella* or *Moraxella*-like, *Flavobacterium* or *Flavobacterium*-like, and *Acinetobacter* or *Acinetobacter*-like. The opportunistic fish pathogens *Aeromonas hydrophila* and *Pseudomonas fluorescens* occurred in low numbers in the biofilters and trout-rearing water.

Key words: Aquaculture, bacteria, fish culture, fluidized sand biofilter.

Intensive propagation of aquatic vertebrates and invertebrates has been practiced throughout the world for thousands of years. Aquaculture is continuing to expand in introduction of new species and in total product. According to the 1991 Aquaculture Buyers Guide (Homer 1991), 18.2 t of consumable fish products were produced worldwide. Availability of water is a limiting factor in continued growth of aquaculture. Water is used once in many culture systems and then discharged. Therefore, more efficient utilization of water is essential for continued expansion of aquaculture. As an alternative to single use of water semiclosed culture systems were developed. In these systems water is recirculated, oxygen is added, and ammonia is removed with a biofilter. Only about 5 to 10% of fresh water is continually added. Because of the need for pumping, addition of oxygen, and other factors, production costs are greater with these systems. Research is being done in many areas to develop more efficient systems. At present the Conservation Fund supports research on a semiclosed trout culture system at the Freshwater Institute, Shepherdstown, West Virginia, through a grant from the Agricultural Research Service of the U.S. Department of Agriculture. Parameters such as the effects of density and loading rates and feeding rates of various diets on trout growth and performance of the fluidized sand biofilter are being evaluated in the system.

An integral part of the semiclosed culture system is the fluidized sand biofilter. Although the main design function of the biofilter is conversion of ammonia to nitrate by the autotrophic nitrifying bacteria, other types of bacteria are also present. We conducted a literature search to determine what had been published on these bacteria, and although studies were found on aquarium sand filters, no studies dealing with fluidized biofilters were found. Therefore, a study was initiated to determine the numbers and types of heterotrophic bacteria that occur in the sand biofilter. Specifically, the numbers and types of heterotrophic bacteria in both systems were studied over time. In addition to determining total heterotrophic bacteria, the study was designed to detect the presence of the opportunistic fish pathogens *Aeromonas hydrophila* and *Pseudomonas fluorescens*, as well as denitrifying bacteria.

Materials and Methods

Because no publications were found on the microbiology of fluidized bed biofilters used in aquaculture, procedures for sampling the fluidized bed and determining the microbiological content of the biofilter had to be developed. The first part of the study was carried out to develop samplers, select total count methods, develop procedures for removing bacteria from sand, determine initial counts of heterotrophic bacteria, and select identification methods for biofilter bacteria. The second part included determination of counts of bacteria that occur over time on sand and in the water phase of the filter, and in tank water containing trout. Additional procedures were also tested to identify biofilter bacteria.

Culture System

A schematic drawing of the semiclosed culture system is presented in Fig. 1. There are two systems termed A and B, and each system consists of two 9,957-L rectangular cross-flow culture tanks, 80 mm mechanical filters, pumps, low-head oxygen injection, carbon dioxide stripper, and a fluidized sand biofilter. The cross-flow tank bottoms are rounded, and water enters through holes just above the bottom along one side and exits through similar holes along the other side. This design provides a

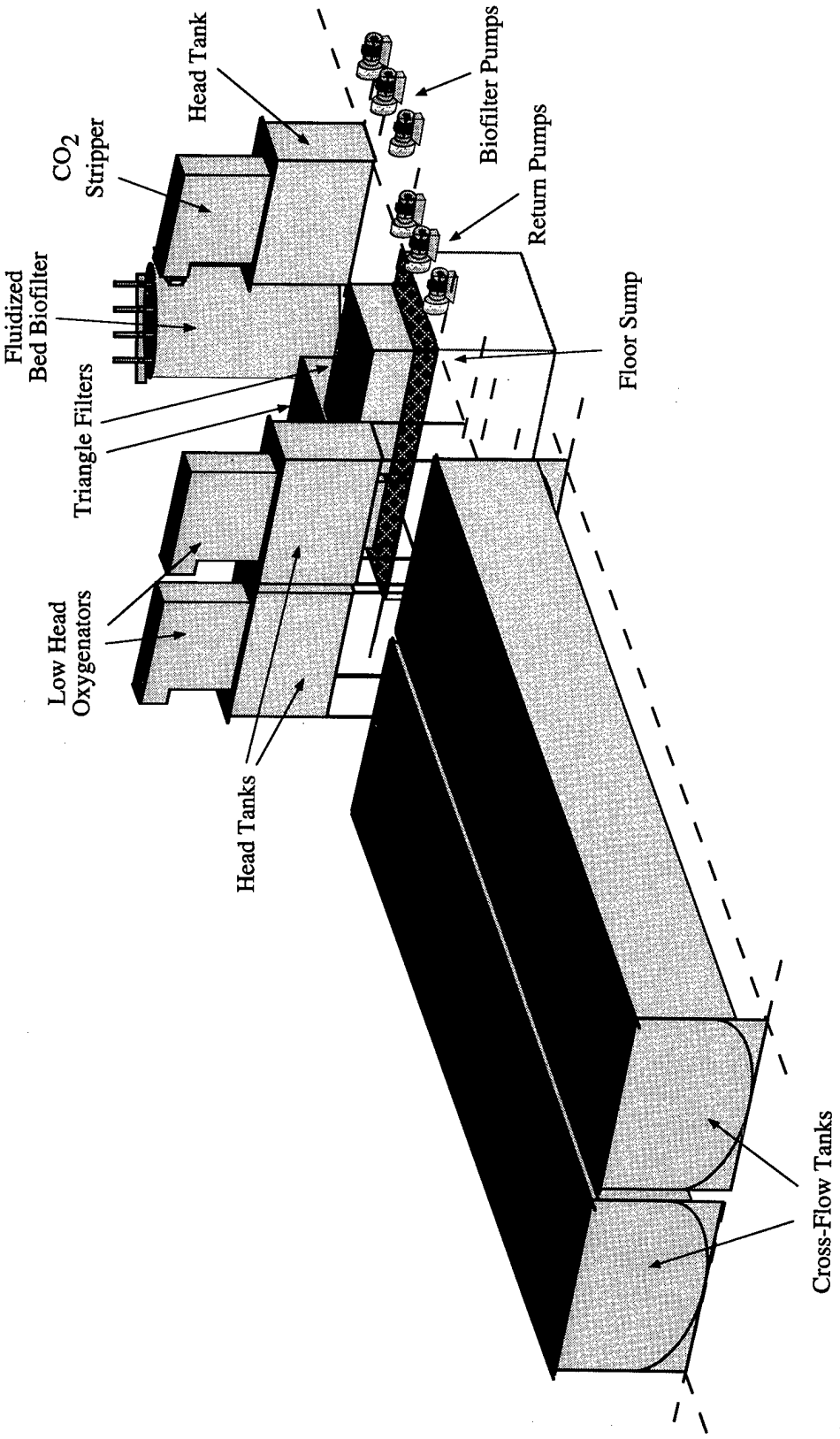

Fig. 1. Schematic diagram of semiclosed trout culture system at the Freshwater Institute, Shepherdstown, West Virginia.

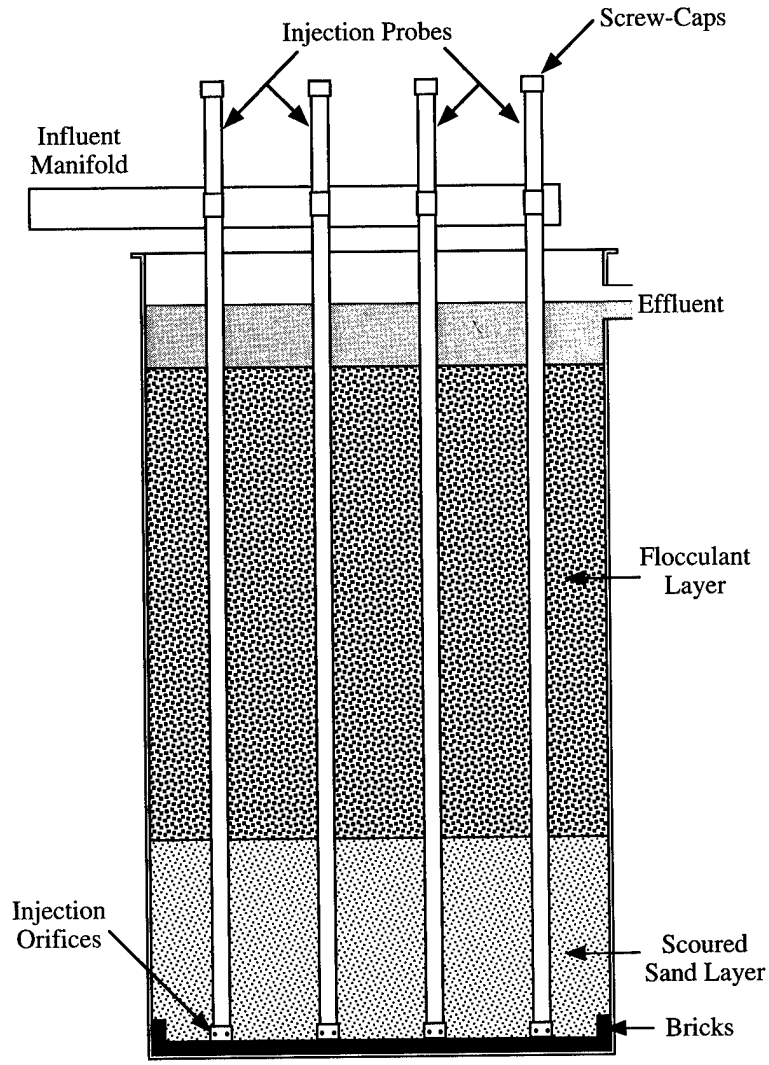

Fig. 2. Schematic diagram of fluidized bed biofilter used in the semiclosed trout culture system at the Freshwater Institute, Shepherdstown, West Virginia.

uniform water quality and self-cleaning of tanks. Incoming water causes tank water to rotate about the long axis of the tank, up-welling on one side and down-welling on the other side, which propels feces and uneaten food toward the outlet. With sufficient numbers of fish no debris settles on the tank bottom, but particles that do not enter exit holes are carried back into the water column creating a constant level of suspended solids. Water flow through the tanks is 314 L/min, which results in a water turnover time of 35 min. Fresh spring water enters tanks at 3 L/min.

Each biofilter (Fig. 2) consists of a circular tank, 2.4 m high and 1.5 m in diameter. Each is filled to about one-third its volume with 212 mm-mesh sand. Water is pumped to the bottom of each biofilter at 757 L/min by means of eleven 3.8-cm polyvinylchloride pipes. Each pipe is capped, and water exits through a ring of lateral holes above the cap. The sand is fluidized as the water rises to the top of the tank. The tank bottom and lower walls are lined with fire brick to protect against sand abrasion. The fluidized bed is maintained 36–51 cm below the top of the tank to prevent sand from washing out. Water temperature throughout the system ranged from 13 to 16° C; dissolved oxygen was 2–6 ppm exiting the biofilter and 8–10 ppm in tanks containing rainbow trout (Oncorhynchus mykiss). Density of trout was one of the parameters studied and varied from 272 to 636 g per 3.78 L. Trout were fed a high-fat grower diet (Zeigler Bros. Inc., Gardners, Pennsylvania).

Samplers

Two samplers were tested to obtain samples at selected depths of the biofilter (Fig. 3). The first consisted of a 125-mL rubber-stoppered,

Fig. 3. Two types of samplers used to obtain biofilter samples from various depths of the filter bed.

sterile serum bottle attached to a 13-mm galvanized pipe. The pipe was lowered to the desired depth, and the rubber stopper was removed from the bottle by using the attached chain (Fig. 3). However, because the serum bottle was not stoppered after the sample was taken, it was possible that the sample could be altered when the bottle was removed from the biofilter. A second sampler was developed that also consisted of a serum bottle attached to conduit pipe, but a screw cap attached to a piece of threaded rod was used to open and close the bottle (Fig. 3). The sampler was lowered to the desired depth, the cap removed, the sample taken, and the cap replaced before removing the bottle. This sampler ensured that the sample taken was representative of the selected depth. Initially, we took samples from depths of 0.9, 1.2, 1.8, and 2.4 m. Preliminary tests showed significant differences only between the 0.9- and 2.4-m samples, so sampling was limited to these depths.

Even though the biofilter material was fluidized and samples probably could have been taken in any area of the filter, all samples, regardless of depth, were taken near the center of the 1.5-m-diameter tank. The proportion of sand and water was measured for each sample.

Plate Count Methods and Media Used

The following procedure was used to determine plate counts from biofilter samples. Immediately after taking a sample, about 5 mL of the water

phase was aseptically transferred to a sterile 13- × 100-mm tube. Samples were immediately placed on ice for transport to the laboratory. Water was decanted, and 40 g of wet sand was aseptically weighed to a sterile 50-mL tube and centrifuged at 5°C for 10 min at 1,000 × g. Water was again decanted, and a 1-g packed sand sample was weighed aseptically. After various treatments described below were applied to the sand, 10-fold dilutions were prepared in pH 7.2 phosphate-buffered saline (PBS), and plate counts were performed. Similar 10-fold dilutions were prepared and plated from the water phase of each sample and from tank water containing trout. The drop plate technique of Miles et al. (1938) and a spread plate procedure (Conn 1957) were compared for determining total heterotrophic bacteria. In the drop plate method six 50-µL drops from selected dilutions were placed on a 15- × 100-mm culture plate. The liquid was allowed to soak in, and colonies within the drops were counted after incubation for 3 or 6 days at 28° C. With the spread plate technique, one 50-µL drop was placed on each of three plates for each dilution. A sterile, bent glass rod was used to spread the drop evenly over the plate, and total colonies per plate were counted and averaged after incubation at 28° C for 3 or 6 days.

For determining total counts of heterotrophic bacteria, brain heart infusion agar (BHIA) and plate count agar (PCA; Difco Laboratories, Detroit, Michigan) were used in studies on system B. We later found that a medium, R2A, had been designed for determining counts of heterotrophic bacteria from water (Reasoner and Geldreich 1985). Higher counts were obtained with this medium, and it replaced BHIA and PCA in studies on system A. The following media were also employed in sampling systems A and B: Rimler Shotts medium (Shotts and Rimler 1973), to detect the opportunistic fish pathogen *A. hydrophila*; pseudomonas isolation agar (PIA; Difco Laboratories), to detect pseudomonads; and nitrate sucrose agar (Rodina 1972), to detect denitrifying bacteria.

Removal of Bacteria From Biofilter Sand

Because bacteria populate surfaces in a flowing system (Blenkinsopp and Costerton 1991), we assumed that biofilter sand would be colonized. The attached bacteria exist within a glycocalyx of extracellular polymeric substance produced by the bacteria (Costerton et al. 1981). To enumerate total heterotrophic bacteria, an effective procedure was needed to remove bacteria from the sand. Initially, we tried three procedures: sonication, mixing on a vortex mixer, and grinding sand by using a mortar and pestle. One-gram wet-packed sand samples were used in the procedures. For sonication, 5 mL of PBS were added to tubes containing sand, and samples were sonicated for 0.5, 1, 2, or 4 min on a Cole Palmer Series 4710, 50-W sonic homogenizer (Cole Palmer Instruments, Chicago, Illinois). For samples mixed on a vortex mixer, 5 mL of PBS were added, and samples were mixed for 1 min at full power. Samples weighed in a mortar were ground for 1 min, and 5 mL of PBS were added to the mortar. After sonication, mixing with the vortex mixer, or grinding, 4 mL of PBS were then added to tubes or mortar to prepare the initial 1:10 dilution. Additional 10-fold dilutions were prepared by using 0.5-mL dilution and freshly prepared 4.5-mL PBS dilution blanks. Total counts were then performed.

In addition to physical means of removing bacteria from sand, three detergents and six enzymes were also tested. Five milliliters of sterile PBS containing 0.1% Triton X-100, sodium dodecyl sulphate (Sigma Chemical Co., St. Louis, Missouri), or Nonidet p40 (Particle Data Lab. Inc. LTD., Elmhurst, Illinois) detergent were added to 1-g sand samples. An additional 4 mL of PBS were added to each tube, samples were vortexed and allowed to react 10 min, dilutions were prepared and counts performed. The following enzymes (Sigma Chemical Co.), each dissolved in 5 mL of PBS, were tested to determine if any would break down biofilm material and remove bacteria from sand: 500 units of alpha glucosidase, 250 units of beta glucosidase, 25 units of alpha galactosidase, 25 units of beta galactosidase, 25,000 units of glucuronidase, 250 units each of alpha and beta glucosidase, and 25 units each of alpha and beta galactosidase. Enzyme mixtures were allowed to react 45 min at 20–22°C, 4 mL of PBS were added to each tube, additional 10-fold dilutions were prepared, and counts were performed.

Identification of Bacteria From Biofilters and Rearing-tank Water

The most predominant colony types were selected from R2A, BHIA, PCA, PIA, and nitrate sucrose plates inoculated with preparations from sand and water from the top and bottom of the biofilter and rearing water containing trout. Each colony selected was streaked onto BHIA, PCA, or R2A to ensure purity, and single colonies were then inoculated onto slants of the medium

from which they were isolated. We selected 220 isolates from system B during nine sampling periods and 128 isolates from system A during five sampling periods. Isolates from system B were identified by using the identification scheme of Weaver (Koneman et al. 1983) and the Oxiferm procedure (Hoffman LaRoche Inc., Nutley, New Jersey). The Weaver scheme uses reaction of isolates with glucose, cytochrome oxidase, and growth on MacConkey agar to place organisms in one of eight groups. Additional tests are then used to identify bacteria to genus and species. The Oxiferm procedure uses results of reactions in nine tests to generate a four-digit number for use in a computer-generated system to identify isolates to genus or genus and species. Because these schemes were designed to identify clinical isolates from warm-blooded animals, many isolates from system B could not be identified. Isolates from system A were identified by using the scheme of Ward et al. (1986), which was designed to identify isolates from water. This scheme uses results from nine tests to generate a three-digit number for identification of bacteria to the genus level. Additional tests are used to identify isolates to species. For those bacteria that could not be identified to genus there is a section for identification to the "genus-like" level.

Sampling From Systems A and B

Studies to determine the best sampling procedure, plate count methods and media, removal of bacteria from the biofilter sand, and initial counts of heterotrophic bacteria *A. hydrophila*, and denitrifying bacteria were carried out with samples from system B. Longer-term studies on variations of the above bacteria over time were carried out with weekly, or at times bimonthly, samples from system A from 23 October 1991 through 26 February 1992. Where appropriate, results were analyzed statistically.

Results

Biofilter sand from systems A and B contained 100–1,000 times more heterotrophic bacteria than did the water phase at the top or bottom of the biofilters. Total number of bacteria on sand at the top of the biofilter was at least 10 times more than

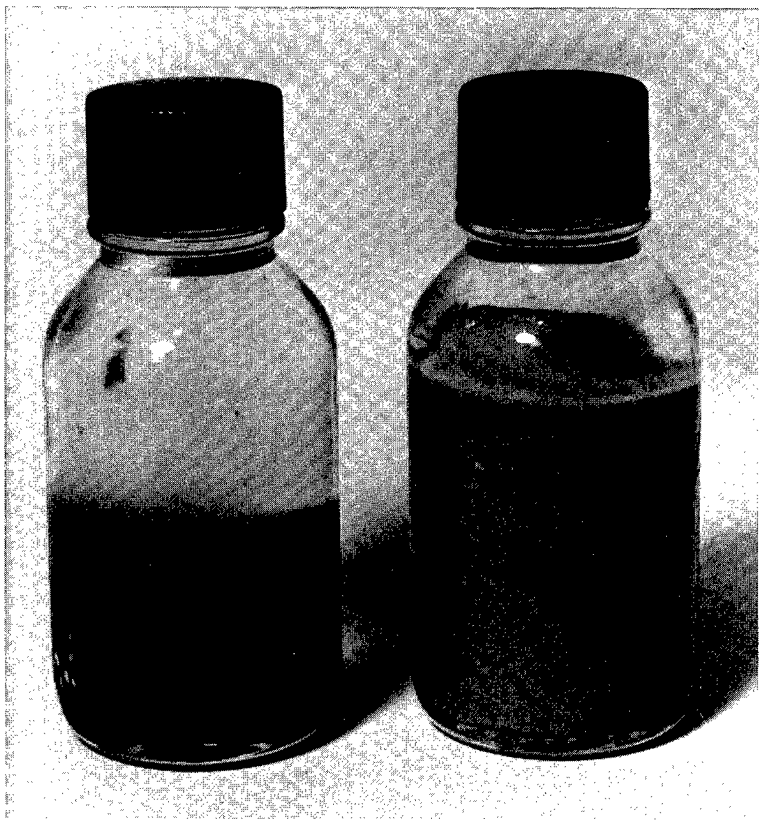

Fig. 4. Biofilter samples from the top (*left*) and bottom (*right*) of the filter bed.

on sand at the bottom of the filter. Neither *A. hydrophila* nor *P. fluorescens* occurred in significant numbers in the biofilters. Predominant bacteria in the biofilters were nonfermenting Gram-negative rods that could only be identified to the genus or genus-like level.

Samplers and Samples

The sampler with the rubber stopper for the serum bottle was used for samples from October 1990 until early March 1991. During this period, methods were being developed for removal of bacteria from sand, making plate counts, and determining incubation times. Although both samplers showed similar counts at various depths, the sampler with the screw cap was used from early March 1991 until the end of the study to ensure that samples were representative of depth.

The physical appearance of samples from the top of the biofilters varied from that of samples from the bottom (Fig. 4). Samples from the top layer were dirty brown with a thin flocculent surface layer that in some samples contained oligochaetes. Samples from the bottom of the filter were white to light gray, but there was no flocculent layer or oligochaetes. The sand-to-water ratio of the samples was significantly different ($P = 0.01$); sand composed 35% of the ratio in samples from the top of the filter and 63% from bottom samples (Fig. 4).

Plate Count, Incubation Period, and Media

The drop plate procedure of Miles et al. (1938) could not be used for total counts of bacteria in the biofilter because spreading colonies overgrew slower-growing organisms. The spread plate procedure eliminated this problem and was used throughout the study. We found that a 6-day incubation period at 28° C was required to obtain accurate total counts. Many of the biofilm bacteria grew slowly and could not be detected after 3 days incubation at 28° C. For example, counts on 1-g wet-packed sand samples plated on BHIA were 2.1×10^7 colony forming units (CFU) after 3 days incubation but had increased to 9.6×10^7 CFU per gram after 6 days incubation. Extending the incubation period to 11 days did not increase total counts. Although BHIA and, at times, PCA were used for total counts during the first 5 months of the study, the R2A medium gave counts up to an order of magnitude higher and was used for total counts on system A studies.

Removal of Bacteria From Biofilter Sand

Two trials were conducted to compare sonication, vortex mixing, and grinding for removal of bacteria from biofilm sand particles. The average counts per gram of wet-packed sand for the two sampling periods were 1.0×10^8 CFU for sonication, 4.8×10^7 CFU for vortex mixing, and 3.4×10^7 CFU for grinding. Examination of the data by one-way analysis of variance indicated sonication produced the highest number of bacteria ($P = 0.01$). Increasing sonication time from 1 min to 4 min neither increased nor decreased total counts. Sonicating a sample for 40 s at 90% power on a larger sonicator (350-W Cole Palmer Series 4710 ultrasonic homogenizer) also did not increase total counts. Therefore, the 1-min sonication using the 50-W sonicator was used to remove bacteria throughout the study. A single trial was conducted to compare sonication with detergent or enzyme treatments for removal of bacteria from sand. Detergents were not more efficient than the 1-min sonication (Table 1). Of the seven enzyme treatments only B galactosidase proved as efficient as 1-min sonication in removing bacteria from sand (Table 2).

Table 1. Comparison of sonication and detergent treatments for removing bacteria from biofilter sand.

	Treatment		Total counts per gram of wet-packed sand[a]
Sonication	50% power	1-min exposure	$6.8 \times 10^{7\,A}$
Detergent			
Nonidet p40	0.1% concentration	10-min exposure	$3.2 \times 10^{7\,B}$
Triton X-100	0.1% concentration	10-min exposure	$1.7 \times 10^{7\,C}$
Sodium dodecyl sulfate	0.1% concentration	10-min exposure	$1.4 \times 10^{7\,C}$

[a] Counts with different letters are significantly different ($P = 0.01$) from each other.

Table 2. Comparison of sonication and enzyme treatments for removing bacteria from biofilter sand.

	Treatment		Total counts per gram of wet-packed sand[a]
Sonication	50% power	1-min exposure	6.4×10^7 A
Alpha glucosidase	500 units	45-min exposure	3.8×10^7 B
Beta glucosidase	250 units	45-min exposure	2.8×10^7 B
Alpha galactosidase	25 units	45-min exposure	2.0×10^7 C
Beta galactosidase	25 units	45-min exposure	5.7×10^7 A
Alpha & beta glucosidase	250 units ea	45-min exposure	3.6×10^7 B
Alpha & beta galactosidase	25 units ea	45-min exposure	4.5×10^7 B
Glucuronidase	25,000 units	45-min exposure	2.8×10^7 B

[a] Counts with different letters are significantly different ($P = 0.01$) from each other.

Total Heterotrophic Bacteria

Regardless of the medium used, counts of heterotrophs were 100–1,000 times higher on sand than in the water phase of the two biofilters (Table 3). The consistently higher counts from sand and water from system A reflect the use of R2A medium. Total counts of heterotrophic bacteria in rearing-tank water were similar to those of the water phase of the biofilter. Counts in tank water containing trout ranged from 1.3 × 104 to 1.7 × 10^4/mL in system B and from 2.2 × 104 to 1.2 × 10^5/mL in system A.

In the preliminary studies with system B and longer-term studies with system A we found that weekly counts from sand and water samples may vary significantly (Fig. 5). To correlate these differences with culture conditions we used Pearsons Product Moment Correlation Analysis (Sokal and Rohlf 1981) on the following variables: dissolved oxygen, nitrite nitrogen, and ammonia nitrogen levels entering the biofilter, leaving the biofilter, and the difference between entering and exit levels; pH entering and leaving the biofilter; water temperature of the system; and 24-h, 48-h, and 7-day feed consumption. For statistical analysis, bacterial count data were paired with physical system environmental data only for the date and time of bacteriological sampling. A more extensive data set was available for system water chemistry, feed inputs, oxygen, pH, temperature, and water flow. The following correlations were found ($P = 0.01$). Bacterial counts on sand at the bottom of the filter were correlated with counts on sand at the top, in that as counts on bottom samples increased or decreased the same occurred in top samples (Fig 5). Bottom water counts were correlated with bottom sand counts and with nitrite removal. As more nitrite was removed by the biofilter, counts in bottom water samples increased. Counts on top and bottom sand samples were correlated with total ammonia nitrogen exiting the biofilter (Fig. 6). Counts in water from the trout rearing

Table 3. Total heterotrophic bacteria on sand and in water phases of biofilter systems A & B.

	Sand[a]		Water[b]	
Biofilter	Top	Bottom	Top	Bottom
System A	6.6×10^7 –	2.4×10^7 –	1.8×10^4 –	3.2×10^4 –
15 sample periods	7.0×10^8	2.6×10^8	6.1×10^5	4.7×10^5
10/29/91–2/26/92				
System B	3.4×10^7 –	1.9×10^6 –	2.3×10^4 –	1.1×10^4 –
7 sample periods	3.9×10^8	8.4×10^7	1.5×10^5	1.6×10^4
1/10/91–7/31/91				

[a] Per gram of wet-packed sand.
[b] Per milliliter of water.

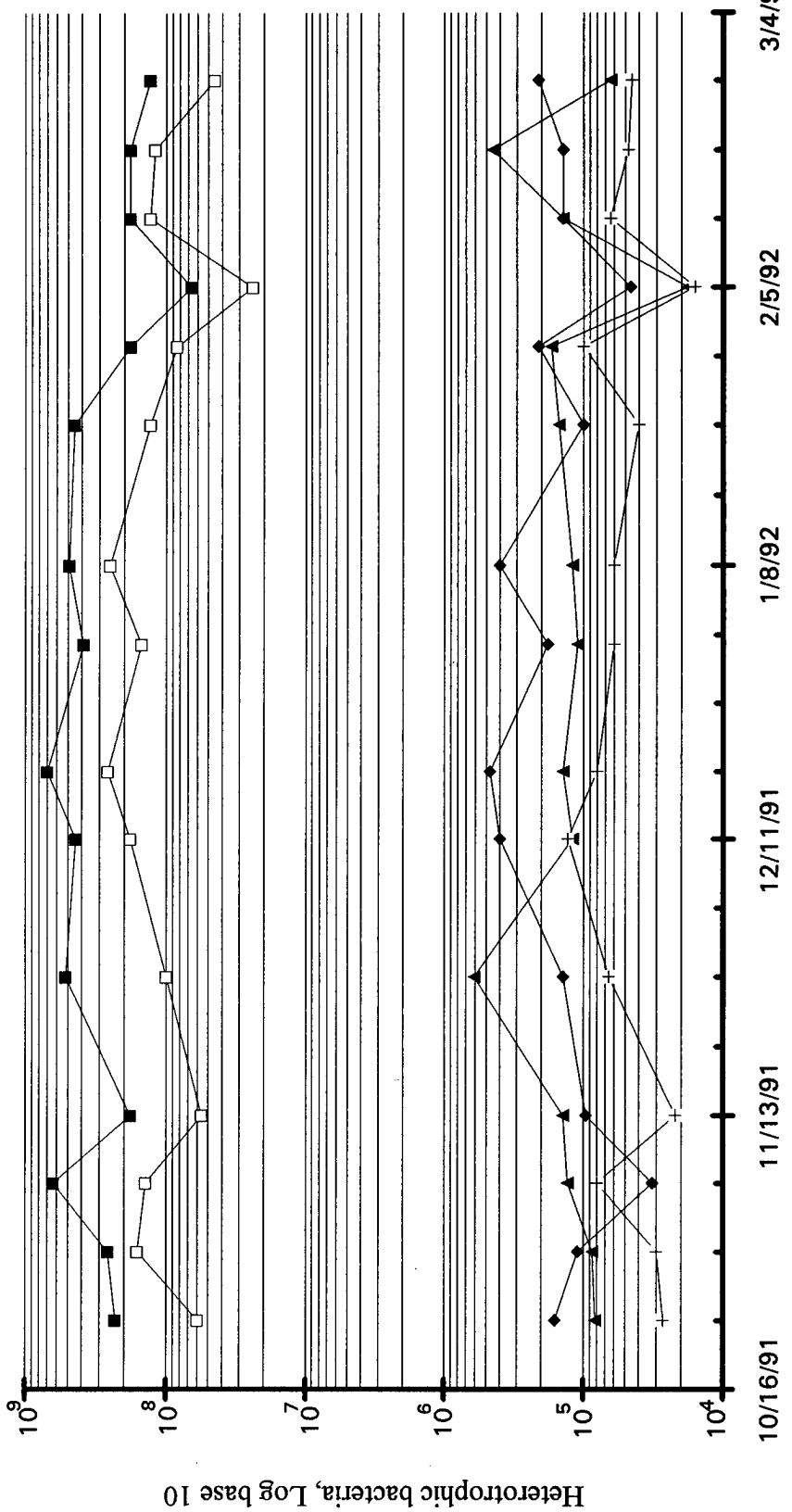

Fig. 5. Total counts of heterotrophic bacteria per gram of sand or milliliter of water from fluidized-bed biofilter using R2A medium. Series identified as sand at the top of the biofilter bed (■), sand at the bottom of the biofilter bed (□), water at the top of the biofilter bed (▲), water at the bottom of the biofilter bed (◆), and water in the rearing tanks (+).

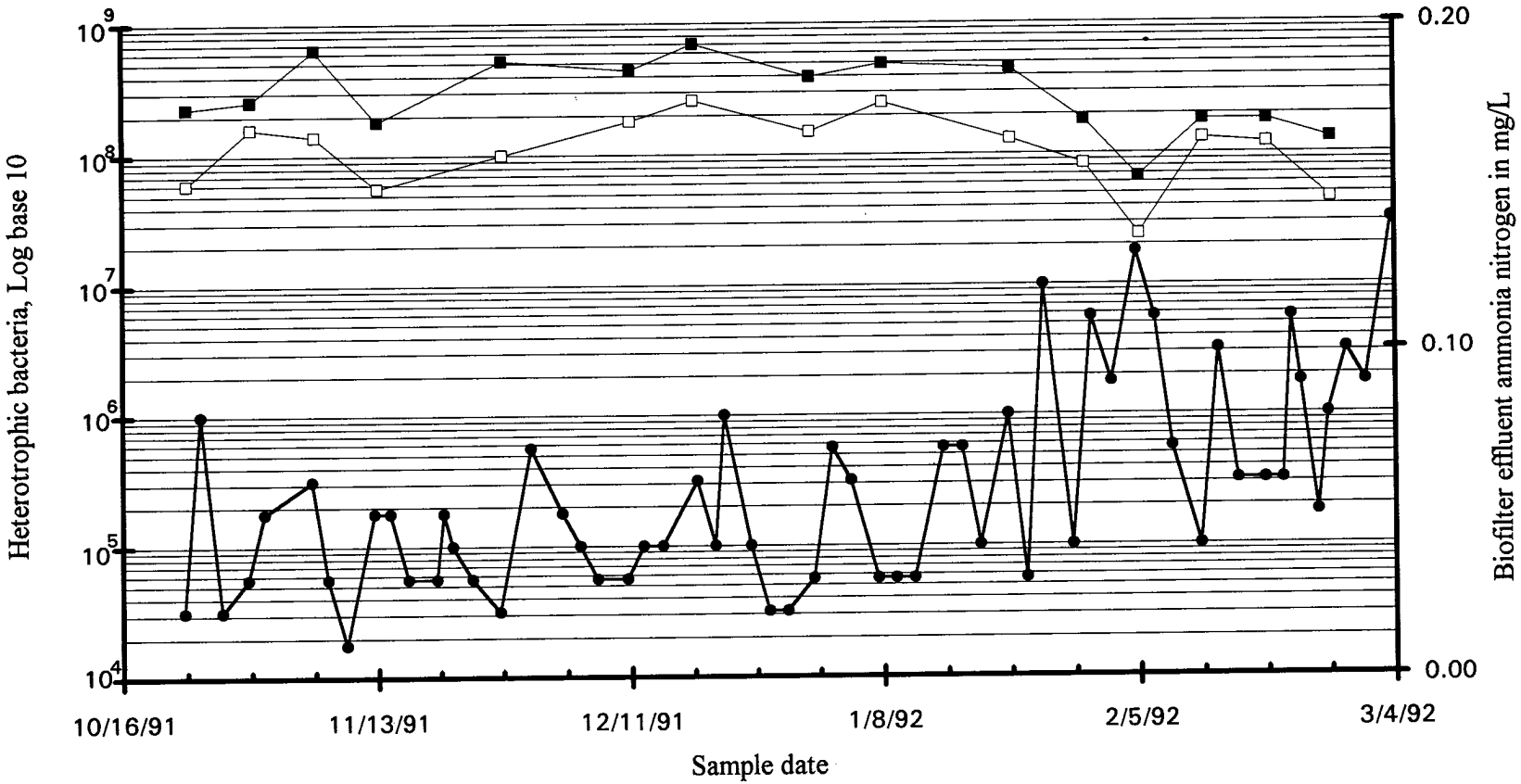

Fig. 6. Fluctuations in total heterotrophic bacteria on sand at the top (■) and bottom (❑) of the biofilter in relation to total ammonia nitrogen (●) measured in the biofilter effluent.

tank were correlated with counts in water at the bottom of the biofilter at the 95% confidence level but not at 99%.

Aeromonas hydrophila

Four attempts were made to isolate *A. hydrophila* from sand and water from system B and two attempts from sand and water from system A. Estimated counts of yellow colonies on Rimler Shotts plates from sand at the top of the filter were only 500 colonies per gram of sand. Fewer than 100 colonies occurred per gram of sand at the bottom of the filter or per milliliter of the water phase of the biofilters or rearing-tank water. Further identification of the various types of yellow colonies on Rimler Shotts plates showed about half were A. hydrophila. Therefore, this opportunistic pathogen did not occur in great numbers in the biofilter or tank water.

Pseudomonads

Highest numbers of pseudomonads in system A during seven sampling periods occurred on biofilter sand. Counts ranging from 1.3×10^6 to 1.6×10^8 CFU per gram were found on sand samples at the top of the filter. Only 1×10^4 to 7.4×10^4 CFU per gram were found on sand samples at the bottom of the filter or per milliliter of water in biofilter and tank water. Most of the isolates from colonies taken from PIA plates were nonfermenting, nonoxidizing pseudomonads. A few colonies were identified as the opportunistic fish pathogen *Pseudomonas fluorescens.*

Denitrifying Bacteria

As with total heterotrophic bacteria counts, more denitrifying bacteria occurred on biofilter sand than in water surrounding the sand or in tank water. Counts on sand samples during seven sampling periods ranged from 1.6×10^6 to 1.6×10^8 CFU per gram of sand at the top or bottom of the filter. Counts of biofilter or tank water samples were 1.3×10^4 to 7.8×10^4 CFU per milliliter. However, these counts do not represent accurate numbers of denitrifying bacteria. When the various colony types were selected from the sucrose nitrate plates and tested for their ability to reduce nitrate, only 50–70% were positive. Growth products from denitrifying bacteria may have provided nitrogen for growth of bacteria unable to utilize nitrate.

Identification of Bacteria in Biofilter and From Tank Water

Of the 274 cultures identified from the two biofilters and rearing tank water 77.1–83.8% were Gram-negative, nonfermenting (GN-NF), rod-shaped bacteria. The yellow-pigmented rods predominated, composing 50% of bacteria in system A and 28.3% in system B. Because the same bacteria occurred on sand and in the water phase

Table 4. Bacteria that occur in biofilters and tank water.

Bacteria	Percentage	
	System A (132 cultures)	System B (142 cultures)
Pseudomonas or *Pseudomonas*-like	11.4	40.7
Moraxella or *Moraxella*-like	15.1	12.1
Acinetobacter or *Acinetobacter*-like	5.3	17.0
Alcaligenes	6.8	6.3
Flavobacterium or *Flavobacterium*-like	8.3	7.0
Flexibacter or *Cytophaga*	6.0	0.7
Aeromonas hydrophila	2.3	9.9
Bordatella	0	1.4
Brucella	0	0.7
Achromobacter	0	0.7
Empedobacter-like	24.2	0.0[a]
Gram-positive cocci or Corynebacteria	13.2	3.5
Unidentified	6.2	0.0

[a] Not included in identification schemes used.

at the top and bottom of filters, we believe isolates occurred uniformly throughout the filters. Identification of the isolates and their percentages in system A and B and in rearing tank water are given in Table 4.

Discussion

Procedures used to determine total heterotrophic bacteria in milk, water, soil, and so forth, could not be used with the fluidized sand biofilter. Bacteria from the biofilm on sand particles must be removed effectively from sand, and a proper plating procedure and culture medium must be used. Also, a 6-day incubation period rather than the traditional 2-day incubation at 28° C is required. Schemes used for identification of GN-NF bacteria isolated from clinical specimens were not useful in identifying isolates from the biofilter. The types of bacteria found in the fluidized sand biofilter were significantly different from those found in rotating biological contactors used in sewage treatment (Antonie and Welch 1969).

A 1-min sonication was more effective for removing bacteria than using a vortex mixer or grinding with sand. Sonication was also more effective than treatment with detergents or six of seven enzyme treatments. Treatment with beta galactosidase was as effective as sonication. Because only one concentration of detergents or enzymes was used to remove bacteria from the biofilm, additional testing of detergents or enzymes might yield higher counts than sonication. Total counts of heterotrophic bacteria on sand or in water did not vary significantly in system A or B in samples from depths of 0.9, 1.23, or 1.82 m, but total counts were significantly lower on sand at the bottom of the filter. This difference was not caused by low oxygen because oxygen is injected at the bottom, but may have been caused by an increased scouring effect of sand particles.

The amount of sand in bottom samples was about twice that found in top samples, and sand from bottom samples was light gray compared with dirty brown in top samples. In studies on the density of the biofilter sand (to be reported in another publication) biofilter sand occurred in two layers differing in density and appearance. Sand at the bottom was completely fluidized and contained larger grains. These conditions resulted in scouring of sand particles, which may have caused the decreased bacterial counts and lighter color of sand compared with sand at the top of the filter. In contrast, sand at the top of the filter was not completely fluidized, allowing a thick biofilm, presence of organic flocculent material, and higher bacterial counts. The uniformity of counts in the filter bed is different from that found in the sand aquarium filter; total heterotrophic bacteria decreased dramatically within 2.5 cm depth of the sand (Kawai et al. 1965). This reduction is associated with decreased oxygen levels. The uniformity of bacterial numbers at various biofilter depths may be attributed to the fluidization of the sand.

Plating procedure, type of media used, and incubation time were important in determining counts of heterotrophic bacteria. The drop plate procedure of Miles et al. (1938) is convenient and accurate, and it requires fewer plates than other procedures. However, because of the slow growth of many bacteria in the biofilter and the presence of spreading yellow-pigmented bacteria, this procedure could not be used. The spread plate procedure worked well and was preferred to the pour plate method because temperature of liquid media (44–45° C) used in this method is lethal to some bacteria occurring in water. Choice of medium is important for enumerating bacteria from water. Reasoner and Geldreich (1985) developed the R2A medium for this purpose. This medium contains several sources of nitrogen, glucose, and starch; however, concentration of these ingredients is at least 10 times lower than in standard media such as BHIA or PCA. The reasoning for lower concentrations of ingredients in R2A medium is that bacteria occurring in water have adapted to an environment with low nutrient concentrations (Reasoner and Geldreich 1985). The lower concentration of nutrients also favors growth of yellow-pigmented bacteria, which composed most of the bacteria found in the biofilters. Extended incubation times of 6 days (Means et al. 1981) were found to give higher counts of heterotrophs in the biofilters.

The counts of bacteria occurring on sand in this study were as much as 10 times higher than those reported by Kawai et al. (1964) in their studies on an aquarium system using a packed sand biofilter. The higher counts may have been the result of more efficient removal of bacteria by sonication. In both biofilter systems counts fluctuated more (Table 3; Fig. 5) than those reported by Kawai et al. (1964). Fluctuations in the sand samples were consistent in that if counts from sand at the top of the filter went up or down counts from the bottom generally showed the same pattern (Fig. 5). Counts from within the filter or tank water containing trout showed no such pattern. One factor that could

cause such fluctuations is variation in sampling technique. Eliminating variation is not possible; however, we took precautions to reduce it. Sampling was carried out on the same day of the week at the same time of day. Samples were iced immediately and transported to the laboratory within 45 min, and procedures were performed as uniformly as possible. The fact that our sand filter was fluidized, and therefore in constant motion, may have affected the counts because we found that simply mixing sand samples and PBS on a vortex mixer removed about 75% of bacteria from sand. The abrasion caused by fluidization may have been in part responsible for some fluctuations in counts. In the packed-sand filter system in the aquarium studies there was no mixing, and the biofilm was not disturbed. However, fluidization would not explain why counts were down one sampling period and up the next. Of all the variables tested for possible effects on bacterial fluctuations, only total ammonia nitrogen level leaving the biofilter correlated with the fluctuations. As ammonia levels reached 0.1 ppm, total counts on sand and in water in the biofilter decreased. However, a combination of factors may have affected bacterial populations because the amount of nitrite removed in the biofilter affected total bacterial numbers in biofilter water. More sophisticated studies are required to determine the cause of bacterial fluctuations in the fluidized sand biofilter.

Although we obtained total counts of denitrifying bacteria that reached 10^8 per gram of sand, the actual numbers of these bacteria were difficult to determine because testing of individual colonies indicated only about half could reduce nitrate. Accurate determination of denitrifying bacteria would require extensive testing of colony types on the denitrification medium.

The opportunistic fish pathogens *A. hydrophila* and *P. fluorescens* did not occur to any extent in the biofilters or fish tank water. These organisms are common inhabitants of water and can usually be readily isolated from freshwater fish culture systems. However, conditions were not favorable for growth of large numbers of these bacteria in the semiclosed system we studied.

The predominant flora of both biofilters were GN-NF rod-shaped bacteria; however, this cannot be construed to mean that all types of biofilters used in fish culture systems contain these types of bacteria. Although microbiological activities of biofilters used in fish culture have been studied, the types of bacteria in the biofilters have not. The types of bacteria in the biofilm of rotating biological contactors used as biofilters in water treatment facilities have been studied. Antonie and Welch (1969) and other authors reported the filamentous organism *Geotrichium canadidum* and the spore-forming *Bacillus cereus* as most important. *Zoogloea filependula, P. denitrificans, Aerobacter aerogenes,* and *Escherichia coli* were also predominant. These organisms were not found in our studies. Studies on fluidized sand and other types of biofilters used in fish culture would have to be done to determine if the types of bacteria in these filters are similar to those we identified.

A striking feature of the bacterial isolates was the predominance of yellow-pigmented rods. Half of the cultures from system A and more than 25% from system B were yellow-pigmented rods. However, identification of the GN-NF bacteria is difficult. The taxonomy of many of these bacteria is uncertain because they have been studied superficially. Many microbiologists tend to place isolates into loosely defined groups, which provides only limited information about these bacteria (Ward et al. 1986). We encountered difficulty in identification of our isolates and could at best identify them to genus. Particular difficulty was encountered with the Oxiferm system and the scheme of Pickett (Koneman et al. 1983) because both are designed to identify isolates from clinical homoiotherm specimens, which have been studied more extensively than those from water. Of 220 isolates selected from system B, only 132 could be identified. We obtained better results with isolates from system A using the scheme of Ward et al. (1986), which was designed to identify GN-NF bacteria from potable water. However, even with this improved procedure many of our isolates failed to produce a coding number for a described organism and had to be identified to genus or genus-like using a coding list for unrecognizable species.

Generally, the same types of bacteria were found in both systems (Table 3), but the percentages varied. For example, 17% of isolates from system B were *Acenitobacter* or *Acenitobacter*-like, whereas only 5.3% occurred in system A. Although 24.2% of isolates in system A were *Empedobacter*-like, no such isolates were identified in system B. This organism was not included in the identification scheme of Pickett (Koneman et al. 1983) or the Oxiferm system, both of which were used to identify organisms from system B. Some organisms identified as *Pseudomonas* in

system B could be *Empedobacter*-like. Additional studies are needed to provide more definitive identification.

To our knowledge, the results reported here represent the first study on a fluidized sand biofilter used in a semiclosed fish culture system for trout. Recently, we were able to examine a fluidized biofilter at the National Fishery Research and Development Laboratory at Wellsboro, Pennsylvania. The bed material of this filter is plastic beads rather than sand. Total heterotrophic bacteria counts from the plastic beads were 10^6 per gram of beads, which is at least 10 times lower than the counts we found on sand. This is not surprising because 1-g sand samples contain more particles than 1-g plastic bead samples and therefore would have more surface area than plastic beads. Identification of the colony types on the plastic beads also showed that GN-NF rod-shaped bacteria were predominant.

Our results raise more questions than they answer. For example, will total heterotrophic bacteria continue to fluctuate on sand and in water over longer periods than we studied, and if they do, what is the cause of fluctuations? Do anaerobic bacteria occur in the filter, and if so, what is their identity? More studies are needed to identify the genus and, if possible, species of the major bacteria. Also, the occurrence of fungi, protozoa, and other organisms needs to be established.

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